BIOTECHNOLOGY-PROCESSES AND PRINCIPLES

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Biotechnology :-

- ✓ **Use of living organism or their products (enzymes) for human usefulness (traditional)**
- ✓ **Processes which use genetically modified organisms for human use on larger scale (modern)**
- ✓ **Example- making bread, curd, wine, IVF/test tube baby, DNA vaccine, synthesizing and using a gene, correcting gene defect etc**
- ✓ **European federation of biotechnology (EFB)- both traditional and modern views**
- ✓ **“Integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services”**

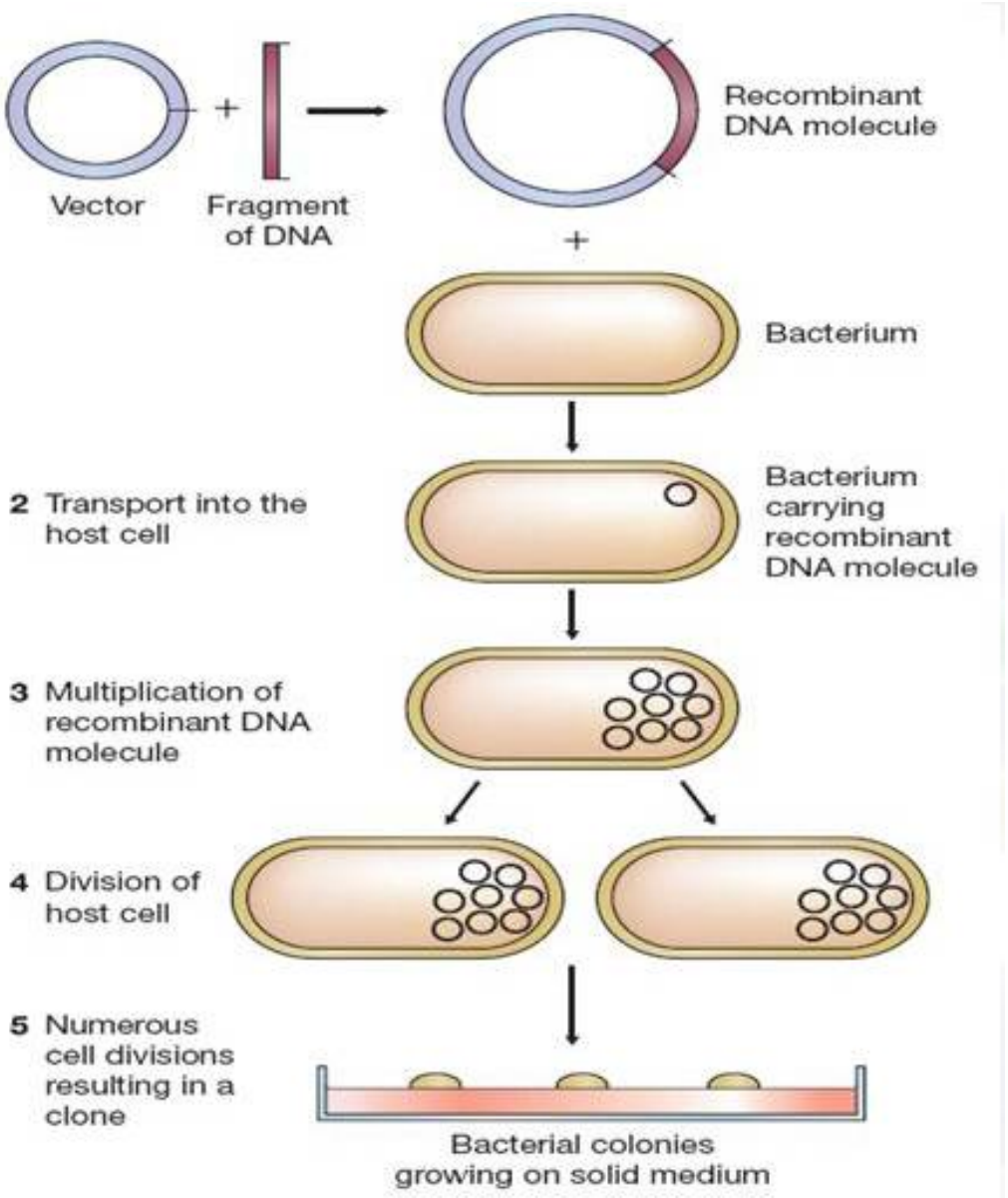
Principles of biotechnology :-

1. **Genetic engineering – techniques to alter chemistry of genetic material (DNA and RNA), to introduce these into host organisms and thus change phenotype of host organism**
2. **Maintainence of sterile conditions- Microbial contamination free ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in large quantity for manufacture of biotechnological products like antibiotics, vaccines, enzymes etc**

- ✓ **Asexual reproduction preserves genetic information**
- ✓ **Sexual reproduction permits variation**
- ✓ **Traditional hybridization procedures in plant/animal breeding –leads to inclusion and multiplication of undesirable genes along with desired genes**
- ✓ **This limitation is overcome by creation of recombinant DNA, use of gene cloning and gene transfer**

Basic Steps in Gene Cloning

1. Construction of a recombinant DNA molecule



Gene cloning

- ✓ **Construction of first rDNA –**
- ✓ **Stanley cohen and Herbert boyer in 1972**
- ✓ **It emerged from possibility of linking a gene encoding antibiotic resistance with a native plasmid (autonomously replicating circular extra – chromosomal DNA) of Salmonella typhimurium**
- ✓ **Cutting of DNA – discovery of "molecular scissors"- restriction enzymes**
- ✓ **Cut piece of DNA was linked with plasmid DNA (act as vectors)**
- ✓ **Plasmid – vector to deliver an alien piece of DNA into host organism**
- ✓ **linking became possible due to enzyme DNA ligase (acts on cut DNA molecules and join their ends)**
- ✓ **rDNA created in – vitro, trasfered into E.coli, could replicate using new host's DNA polymerase enzymes and make multiple copies (gene cloning)**

Three basic steps in genetically modifying an organism

1. **Identification of DNA with desirable genes**
2. **Introduction of identified DNA into host**
3. **Maintenance of introduced DNA in host and transfer of DNA to its progeny**

Tools of rDNA technology

1. **DNA manipulative enzymes**
2. **Vectors**
3. **Competent hosts**

<u>DNA manipulative enzymes</u>

1. **lyases (use to open up cells to get DNA) – cellulase (plant cells), chitinase, (fungus), lysozyme (bacteria), detergents, ribonuclease (RNA removal), protease (protein removal)**
2. **Restriction enzymes / cleaving enzymes -used to cut DNA molecules**
   - ✓ **In 1963 two enzymes ,responsible for restricting growth of bacteriophage in E.coli isolated**

- ✓ **One added methyl groups to DNA, other cut DNA (k/a restriction endonuclease)**
- ✓ **First isolated R.E – Hind II (five years later)**
- ✓ **Always cuts DNA at a particular point by recognizing a specific sequence of six base pairs (recognition sequence for Hind II)**
- ✓ **More then 900 R.E have been isolated from over 230 strains of bacteria, with different recognition sequences**
- ✓ **Nomenclature of R.E**
- ✓ **R.E are obtained from prokariotes. It is there natural defense mechanism against bacteriophage infection**
- ✓ **Each RE recognizes a specific palandromic nucleotide sequences in DNA**

Nomenclature of RE :-

1st letter
(Capital)
– Genus
– Eg. : Escherichia

2nd & 3rd letter
(small)
– Species
– Eg. : Coli

4th letter
(Capital or small)
– Strain of Bacteria
– Eg. : Ry13

5th letter
(Roman numerals)
– Specifies experiment no. or Order of discoverey

- ✓ **Palindrome – In DNA, sequence of base pairs that reads same on two strands when orientation of reading is kept the same**
- ✓ **RE cut each of two strands of double helices DNA at specific points in suger – phosphate backbones**
- ✓ **Sticky ends – over hanging stretches, single stranded portions of DNA at ends , produced due to RE cutting a little away from center of palindrome, but between same two bases on opposite strands**
- ✓ **RE- used in genetic engineering to form rDNA (DNA from different sources/ genomes)**

- **Sticky End Producing RE :-**
  1. **Sal I → Streptomyces albus**
  2. **Bam HI → Bacillus amylolique faciens (G↓GATCC)**
  3. **EcoRI → Escherichia coli RY13 *(G↓AATTC)**
  4. **Hind III → Haemophilus influenzae Rd (A↓AGCTT)**
  5. **Pst I → Providentia Stuartii**
  6. **Pvu I → Proteusr vulagaris**
  7. **Hpa II → Haemophilus paraintluenzae**

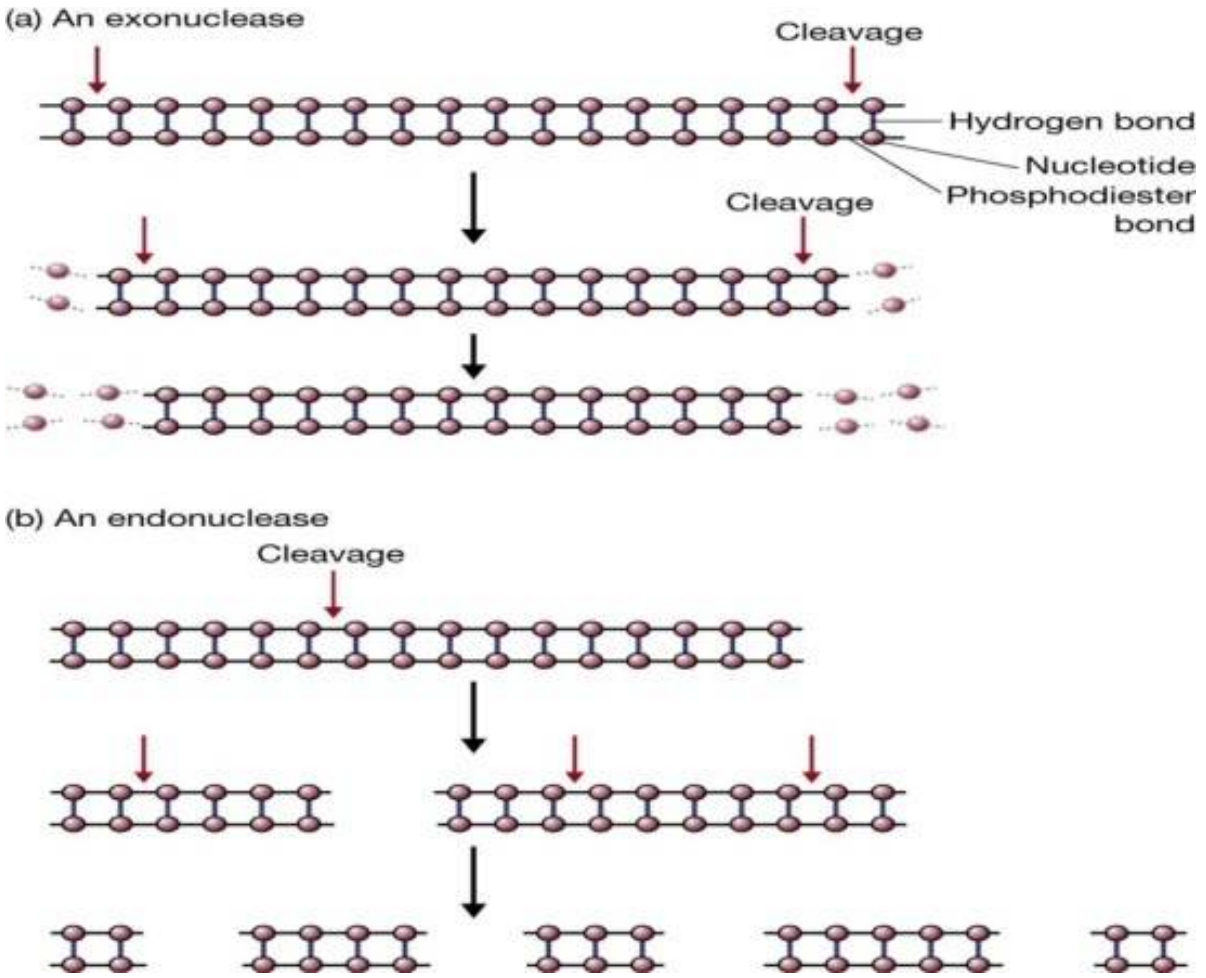
- **Blunt End Producing RE :-**
  1. **Alu I → Arthrobacter luteus**
  2. **Hae III → Haemophilus aegyptius**
  3. **Sma I → Serratia marcescens *(CCC↓GGG)**
  4. **Hind II → Haemophilus influenzae Rd (GTCGAC)**
  5. **EcoRII → Escherichia coli RY 13**
  6. **Sca I → Streptomyces Caespitosus**
  7. **Hpa I → Haemophilus paraintluenzae**

- ✓ **When cut by same RE, DNA fragments with same kinds of sticky ends can be joined together (end - to- end) using DNA ligases**
- ✓ **Unless vector and source DNA is cut with same RE, recombinant vector molecule can not be created**

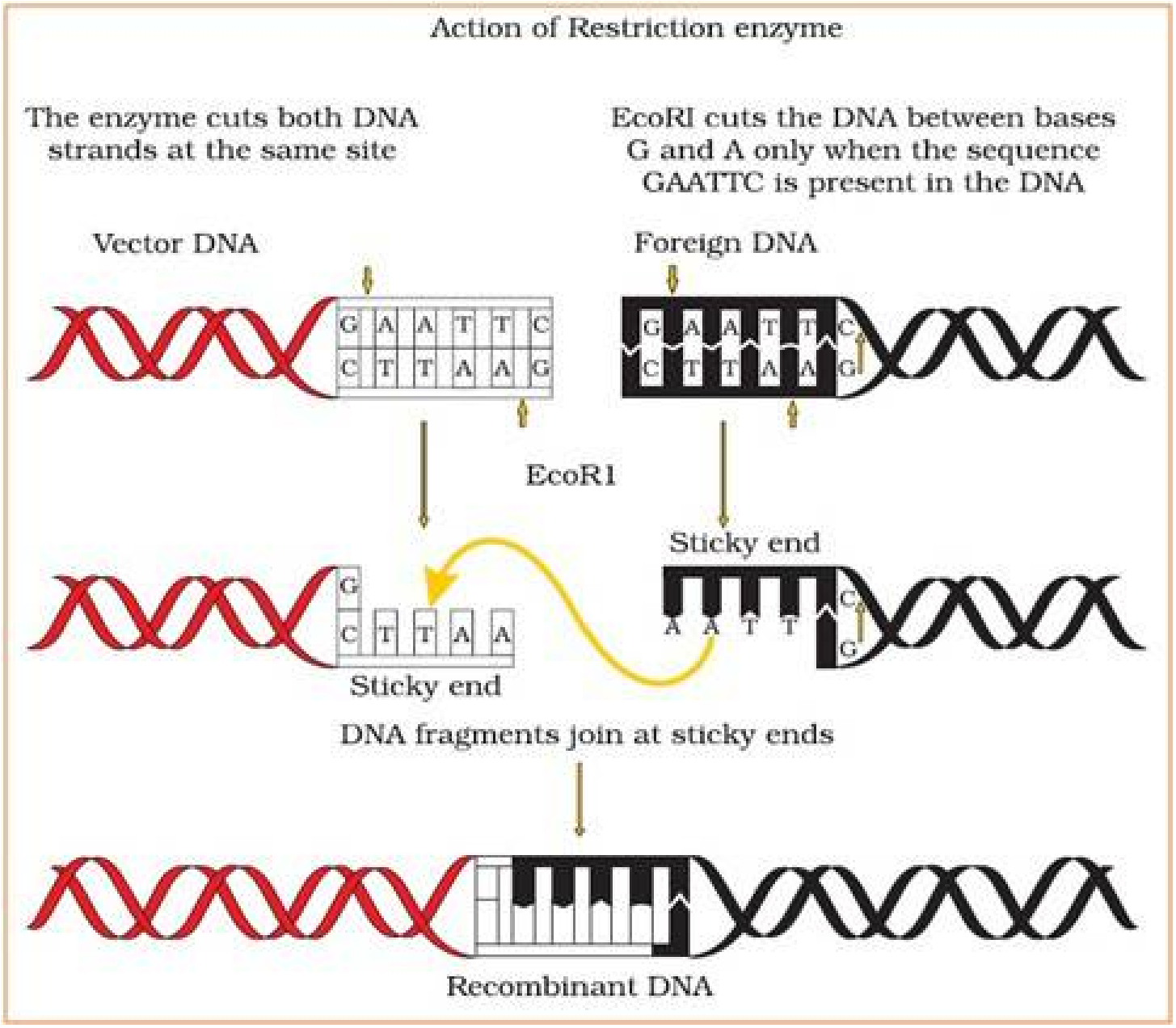
3. **DNA ligases – forms phosphodiester bonds between adjacent nucliotides, covalently links to fragments of ds-DNA, uses energy. EG – T_4 DNA ligase, encoded by phage T_4**
4. **DNA polymerases- synthesize new strand of DNA, complimentary to existing in 5'to 3'direction**

Nucleases –

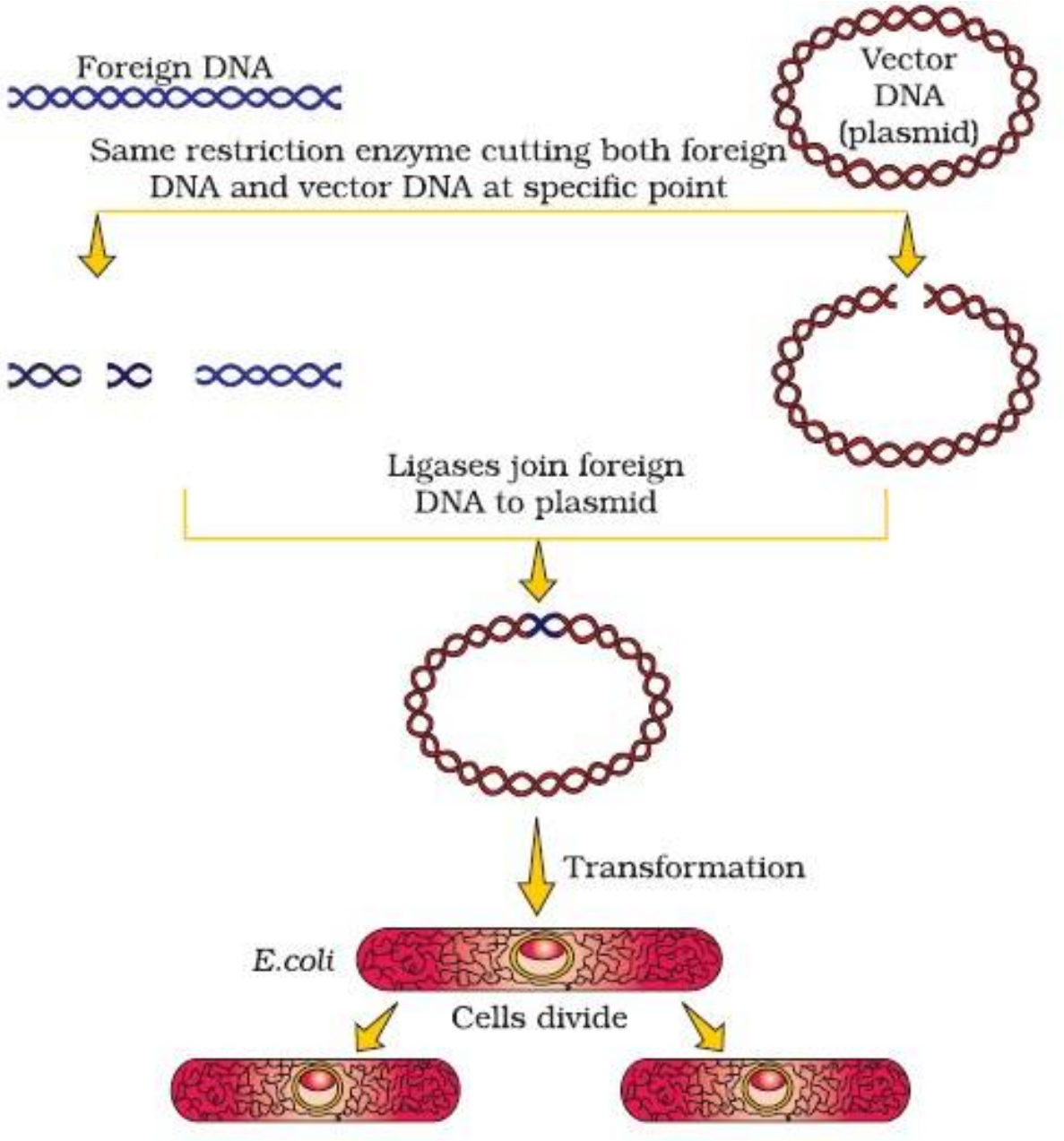
(a) Exonuclease, (b) Endonuclease



The reactions catalyzed by the two different kinds of nuclease.
(a) An exonuclease, which removes nucleotides from the end of a DNA molecule.
(b) An endonuclease, which breaks internal phosphodiester bonds.



Steps in formation of recombinant DNA by action of restriction endonuclease enzyme - EcoRI



Diagrammatic representation of recombinant DNA technology

DNA Ligases

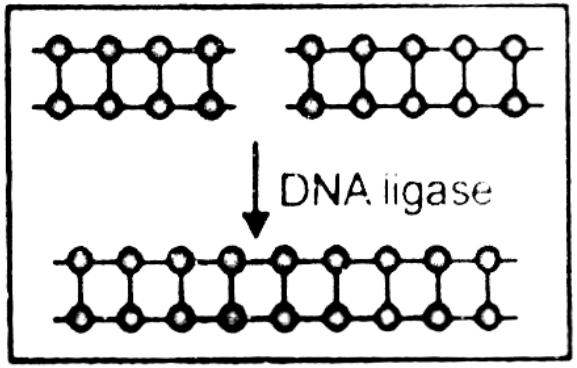


Fig. : Joining two molecules

DNA Polymerases

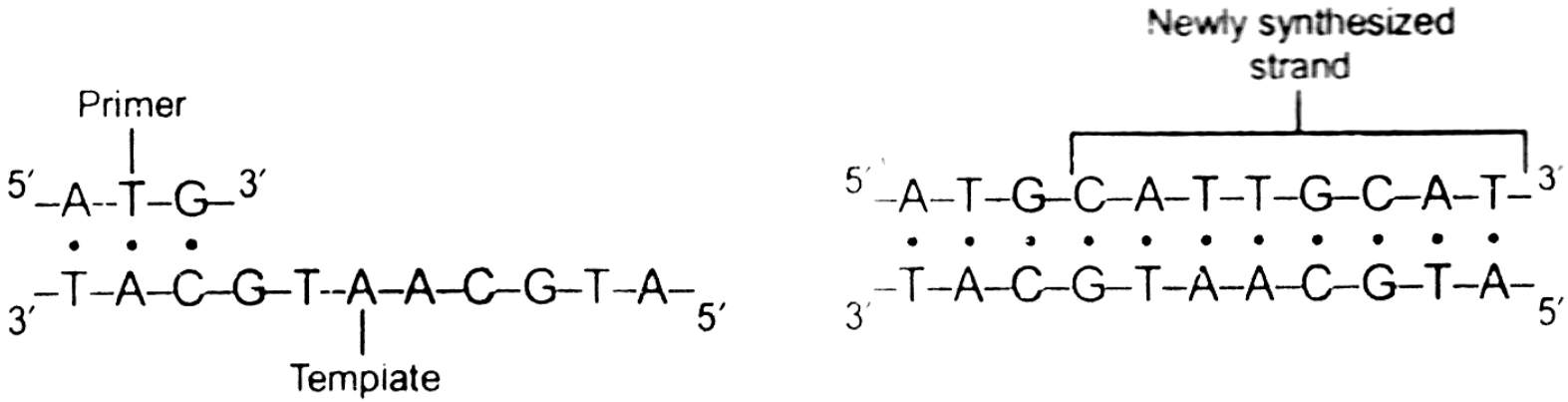


Fig. : Extension reaction

Table : Recognition Sequences of Several Restriction Endonucleases

Enzyme	Microbial origin	Recognition site
Bam HI	*Bacillus amyloliquefaciens*	5′–G↓G–A–T–C–C–3′ 3′–C–C–T–A–G–G↑–5′
Eco RI	*Escherichia coli*	5′–G↓A–A–T–T–C–3′ 3′–C–T–T–A–A–G↑–5′
Hind III	*Haemophilus influenzae*	5′–A↓A–G–C–T–T–3′ 3′–T–T–C–G–A–A↑–5′
Pst I	*Providencia stuartii*	5′–C–T–G–C–A↓G–3′ 3′–G–A–C–G–T–C↑–5′
Sal I	*Streptomyces albus*	5′–G↓T–C–G–A–C–3′ 3′–C–A–G–C–T–G↑–5′
Sma I	*Serratia marcescens*	5′–C–C–C↓G–G–G–3′ 3′–G–G–G–C–C–C↑–5′

Cloning vector

- ✓ **Carrier/vehicle that delivers foreign piece of DNA into host**
- ✓ **Helps in – easy linking of foreign DNA and selection of recombinants from non – recombinants**
- ✓ **Commonly used – bacteriophages and plasmids, have ability to replicate within bacterial cells independent of control of chromosomal DNA**

Essential features

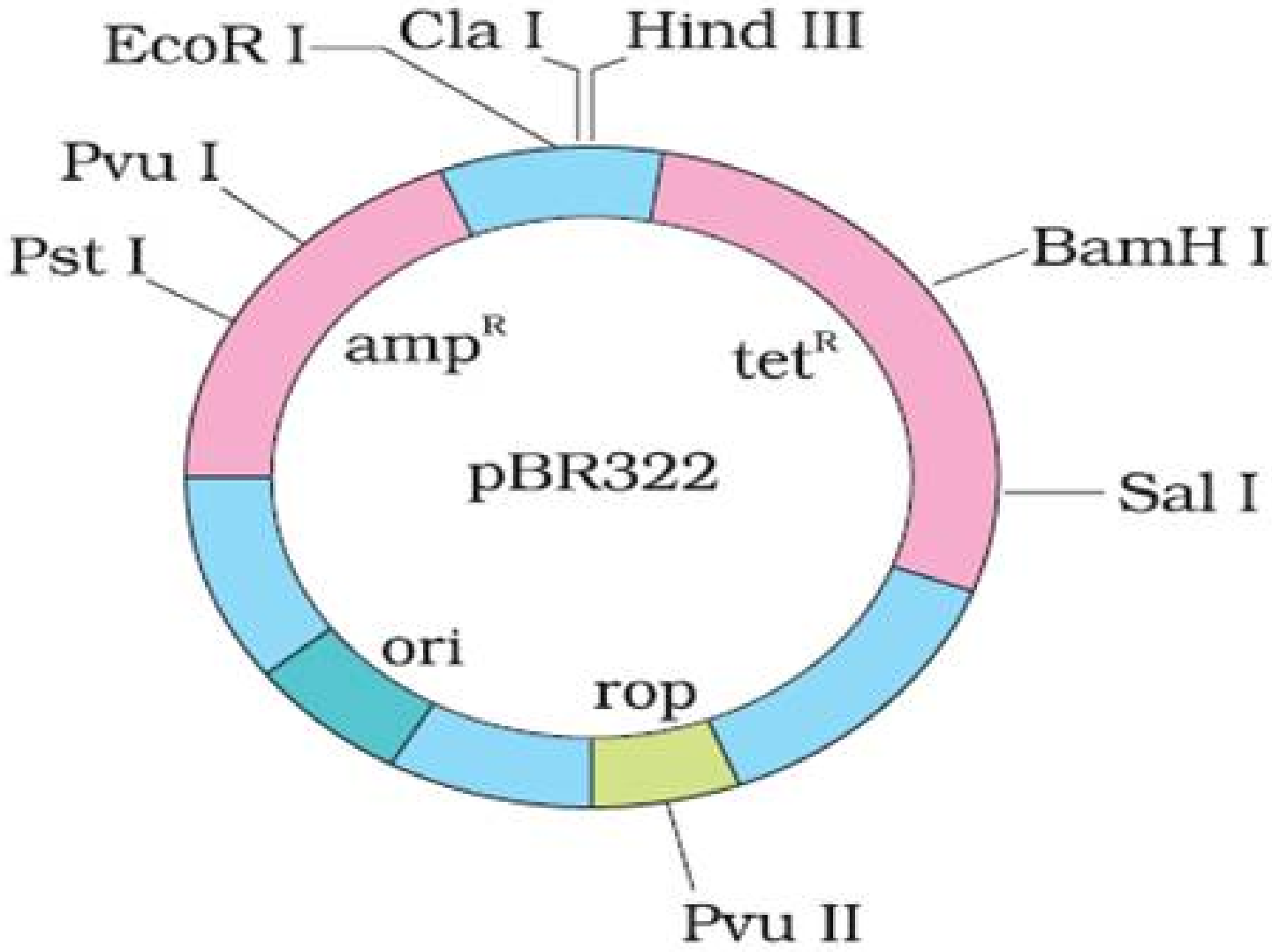
1. **Origin of replication (ori) - sequence from where replication starts**
   - ✓ **Any piece of DNA when linked to ori can be made to replicate within host cells**
   - ✓ **Responsible for controlling copy number of linked DNA**
   - ✓ **If many copies of target DNA to be recovered , should be cloned in vector whose origin support high copy number**
   - ✓ **rop – codes for proteins involved in replication of plasmid**

2. **Selectable marker – identify and eliminate non – transformands and selectively permiting growth of transformant**
   - ✓ **Eg. Genes encoding resistence to antibiotics like ampicillin, chloramphenicol, tetracycline, kanamycin etc for E.coli. (normal E.coli cells do not carry resistance against any of these antibiotics)**
   - ✓ **Gene lac Z coding for β – galactosidase enzyme (utilizes substrate to produce blue colored product)**

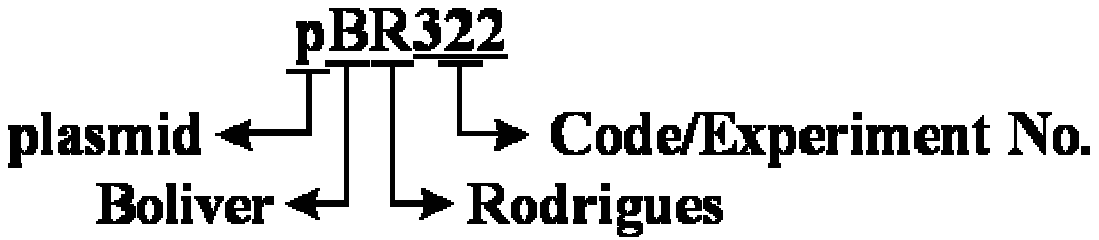
3. **Cloning sites- to link alien DNA ,vector needs to have very few, preferably single recognition sites for RE**
   - ✓ **More then one such sites will generate several fragments and complicate gene cloning**
   - ✓ **Ligation of alien DNA is carried out at a restriction sites present in one of two antibiotic resistant gene /selectable marker genes**
   - ✓ **Recombinant vector will loose antibiotic resistance due to insurgence of foreign DNA – insertional activation**

Examples of vectors-

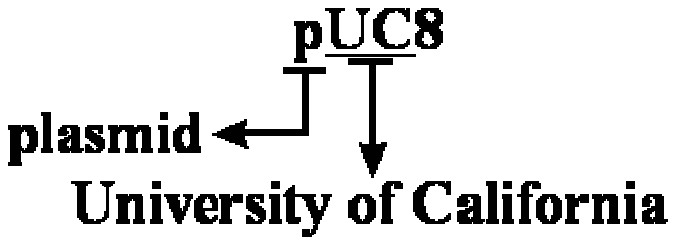
1. **plasmids - extra chromosomal, circular, double stranded, autonomous, self replicating pieces of DNA in bacterial and some yeast cells**
   - ✓ **Some plasmids have only 1-2 copies per cell, others 15-100 copies per cell or even higher**
   - ✓ **pBR322 – plasmid**



E. Coli cloning vector pBR322 showing restriction sites (Hind III, EcoR I, BamH I, Sal I, Pvu II, Pst I, Cla I), ori and antibiotic resistance genes (amp^R and tet^R). Rop codes for replication of the plasmid.



* 1st Artificial Cloning plasmid
* 2 Selectable Markers - amp^r, tet^r
* 1 ori
* 1 rop
* 8 RE sites
* a) Hind III (Haemophilus influenzae)
  b) EcoRI (Escherichia coli RY 13)
  c) Cla I (Caryophanon Latum)
  d) BamHI (Bacillus amyloliquefacien)
  e) Sal I (Streptomyces albus)
  f) Pvu II (Proteus vulgaris)
  g) Pst I (Providentia Stuartii)
  h) Pvu I (Proteus vulgaris)

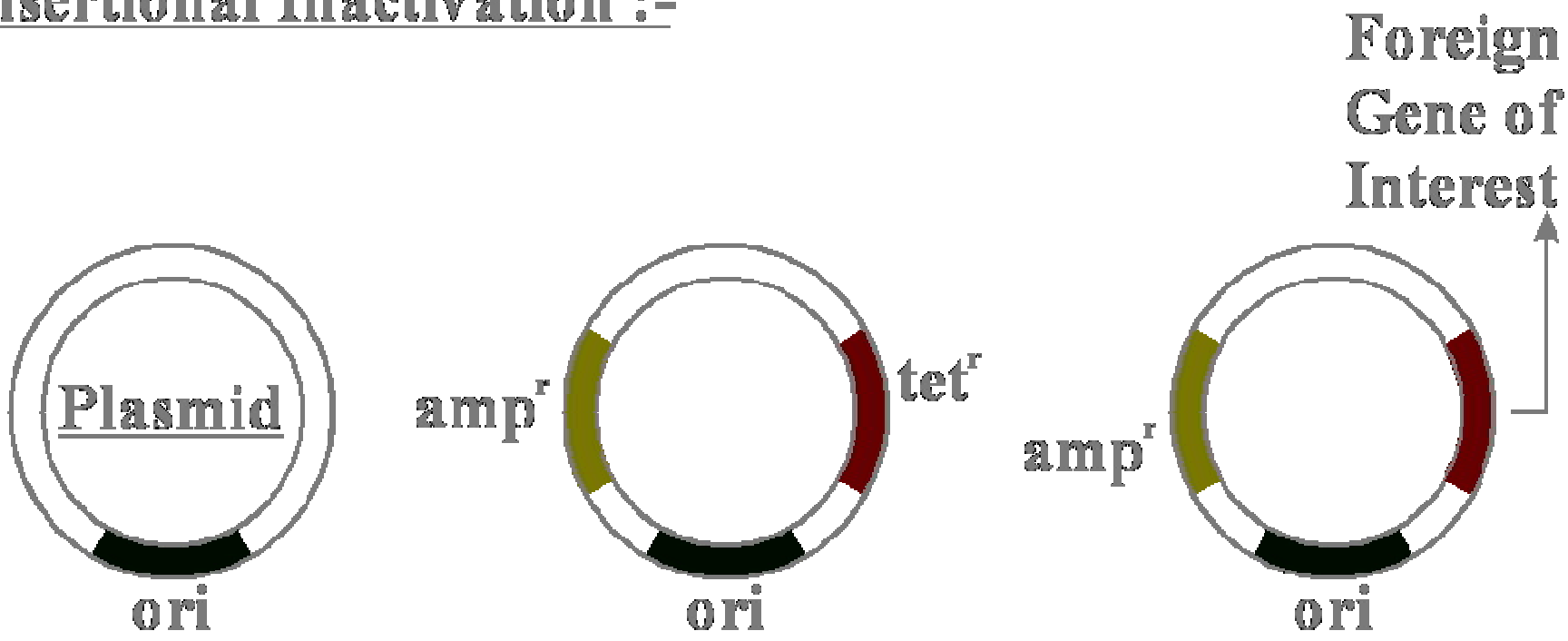


* Marker : Lac Z gene
  Blue - Non-recombin.
  White - Recombinant

	VECTOR	ORGANISMS	INSERT SIZE
1	Plasmid	E. Coli	Upto 15 Kb
2	Phagemid	Bacteriophage	25-35 Kb
3	Cosmid	E. Coli	Upto 45 Kb
4	BAC	Bacteria	200-300 Kb
5	YAC	Yeast	250-1000 Kb
6	HAC	Human	> 1000 Kb

- **Phagemid = Bacteriophage + Plasmid**
- **Cosmid = COS site of Lambda phage + mid from plasmid DNA**
- **Transposons/ mobile/ jumping genes - by Clintock in Maize plants**
- **Shuttle vectors – Eg. Yep (Yeast - episomal Plasmid)**

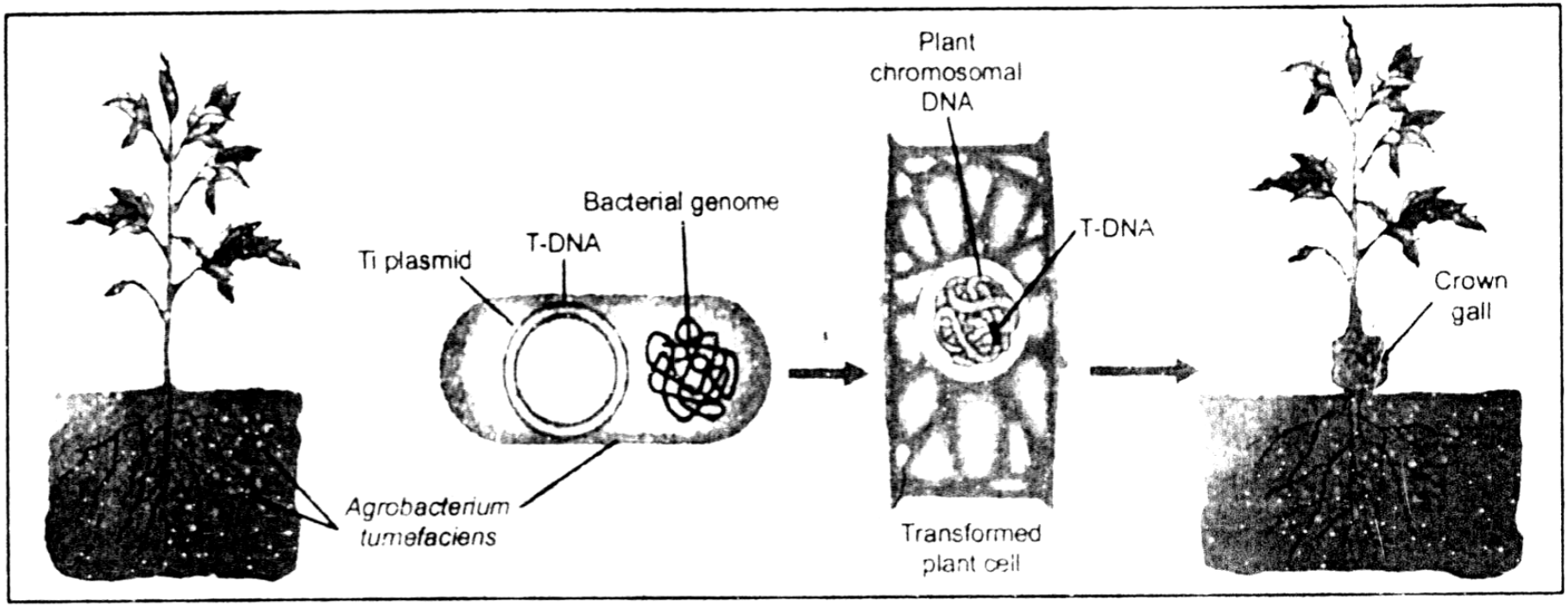
Insertional Inactivation :-



	Non-transformat Non-recombinant	Transformant Non-recombinant	Transformant Recombinant
Medium containing ampicillin	Die	Live	Live
Tetracycline	Die	Live	Die
Both	Die	Live	Die

Vectors for cloning genes in plants –

- **Ti plasmid of Aagro bacterium tumefaciens- pathogen of several dicot plants is able to deliver a piece of DNA (T-DNA) to transform normal plant cells into a tumor and direct these tumot cells to produce chemicals required by pathogen. Form “crown gall tumor”**
- **Modified into a cloning vector, no more pathogenic to plants but still able to deliver gene of interest into plants**
- **Ri plasmid of Agrobacterium rhizogenes – cause “rhizoids”**
- **Vector used in animals – disarmed retroviruses**
- **Shuttle vector – can replicate in both eukaryotic cell and R.coli contains two types of ori and selectable marker genes. eg**


Plant
chromosomal
DNA
Bacterial genome
T-DNA
Ti plasmid
T-DNA
Crown
gall
Agrobacterium
tumefaciens
Transformed
plant cell

Competent host (for transformation with rDNA) –

- **DNA is hydrophilic molecule can't pass through cell membrane. In order to force bacteria to take up the plasmid, first made competent, by treating with specific conc. of a divalent cation, such as calcium, increases efficiency with which DNA enters bacterium through pores in its cell wall**

1. **Transformation by heat shock treatment**
2. **Microinjection – Directly injected into nucleus of animal cell**
3. **Biolistics or gene gun – suitable for plants, high velocity micro particles of gold or tungsten coated with DNA bombarded**
4. **Disarmed pathogen vectors – When allowed to infect the cell, transfer rDNA into host. Eg – Ti plasmid, retroviruses**
5. **Electroporation increase permeability of protoplasts membrane by creating pores**
6. **PEG/polyethylene glycol – Helps in protoplasts fusion, foreign DNA to enter hosts cell**

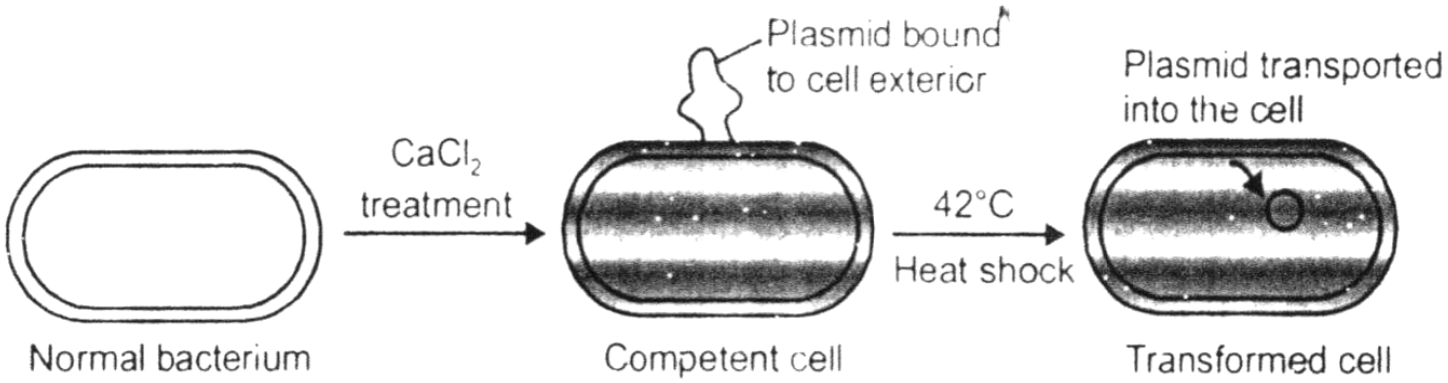


Fig. : The binding and uptake of DNA by a competent bacterial cell through transformation

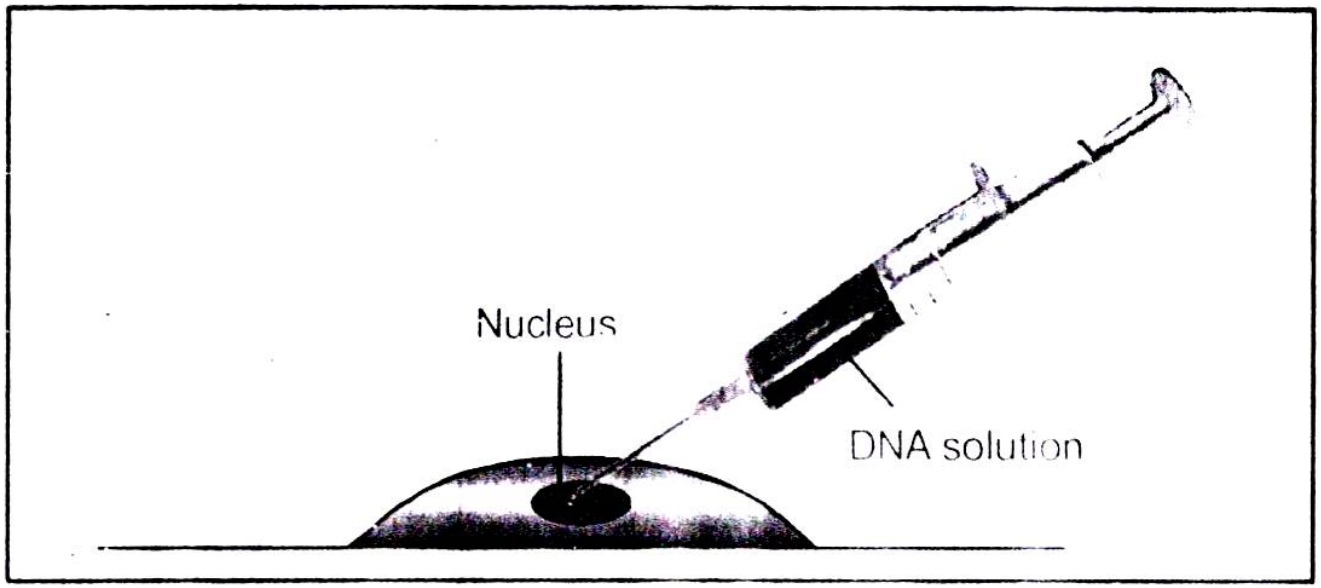


Fig. : Microinjection

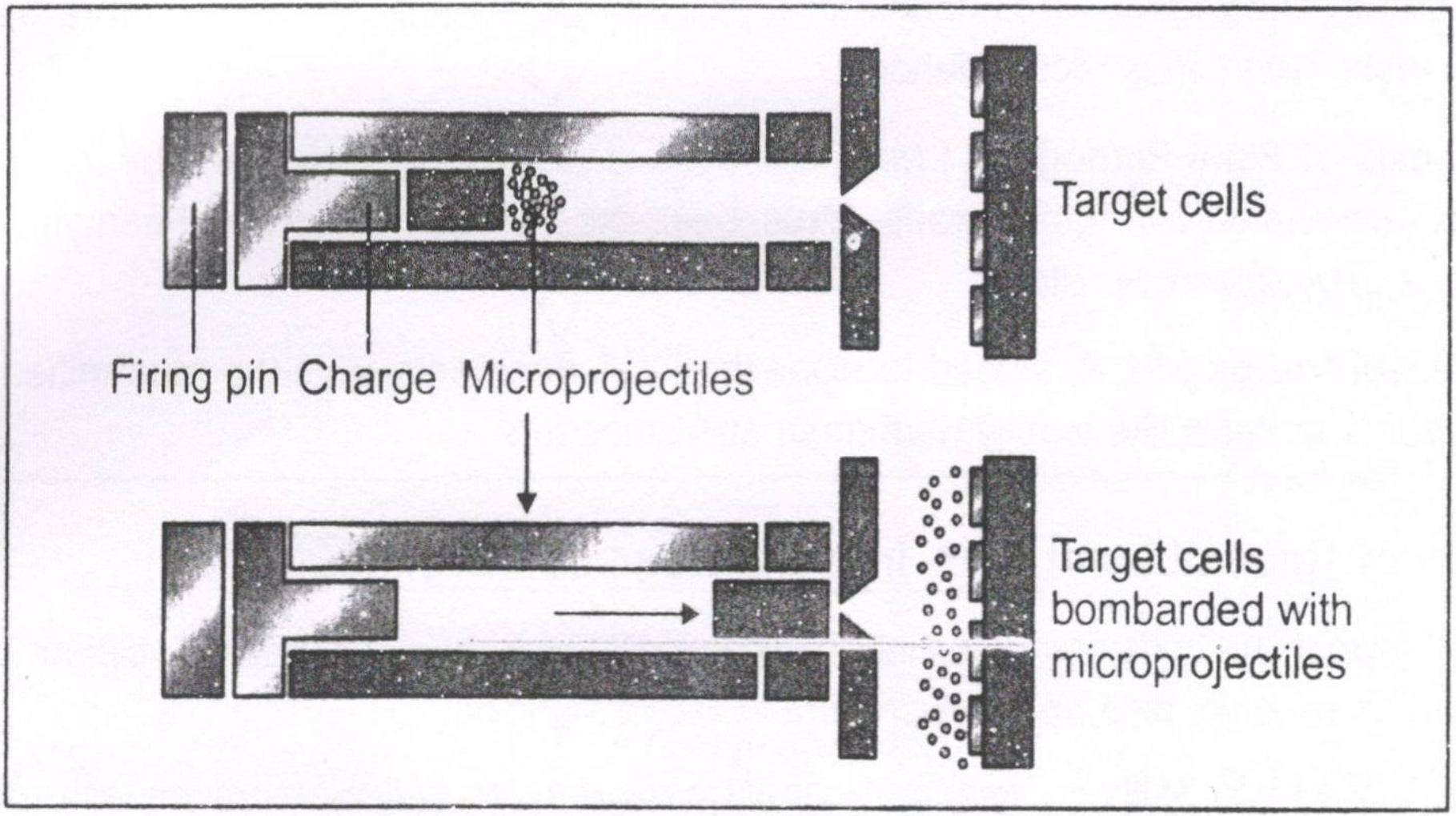


Fig. : Transformation with microprojectiles

Processes of rDNA technology-

1. **Isolation of DNA**
   - ✓ **Cells are broken open to release DNA by lysing enzymes**
   - ✓ **Purified DNA precipitates out by addition of "chilled ethanol". Seen as collection of fine threads in suspension**
   - ✓ **Separated out DNA removed by "spooling "**
2. **Cutting /fragmentation of DNA by RE**
3. **Separation and isolation of desired DNA fragment**
   - ✓ **By gel electrophoresis**
   - ✓ **Since DNA fragments are negatively charged, move towards anode under electric field through a medium /matrix (agarose –natural polymer from sea – weeds)**

3.1 Preparation of total cell DNA

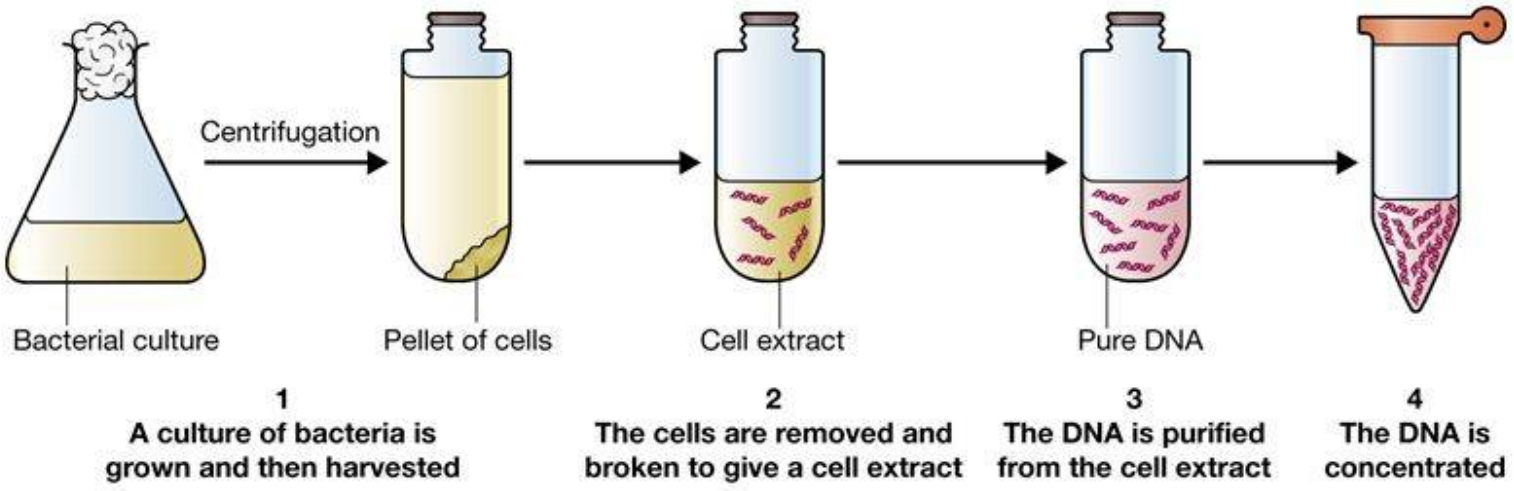
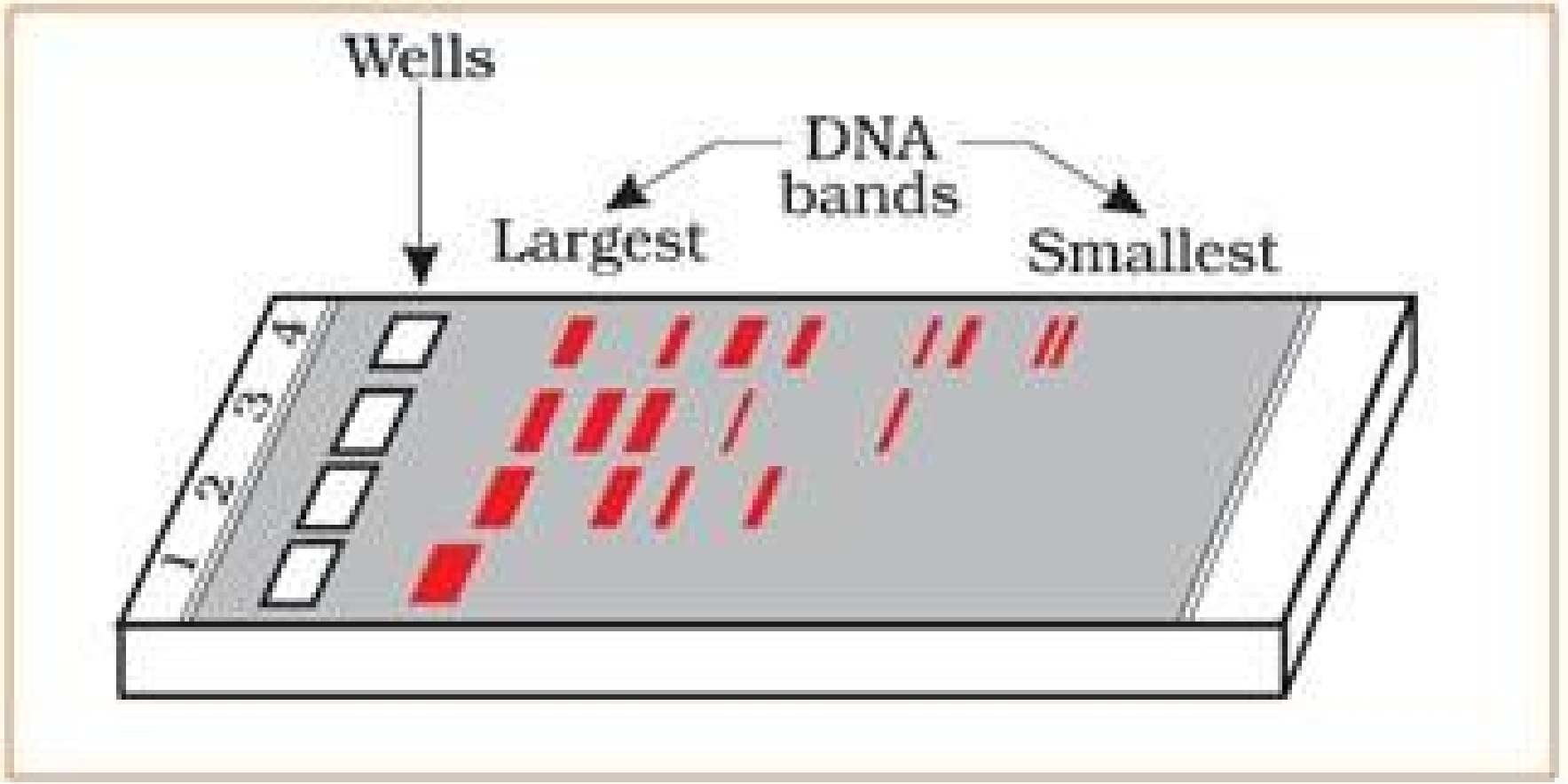
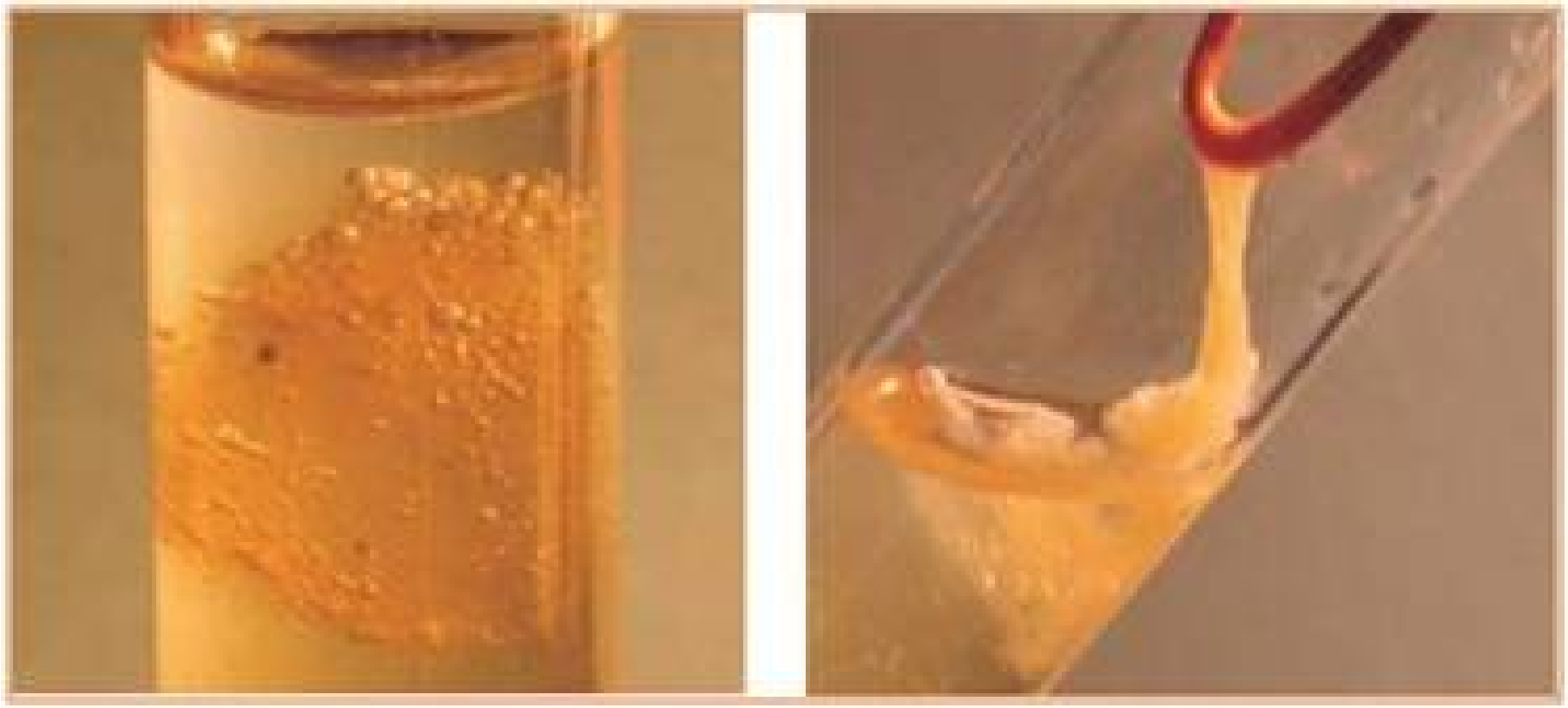


Figure 3.1
The basic steps in preparation of total cell DNA from a culture of bacteria.



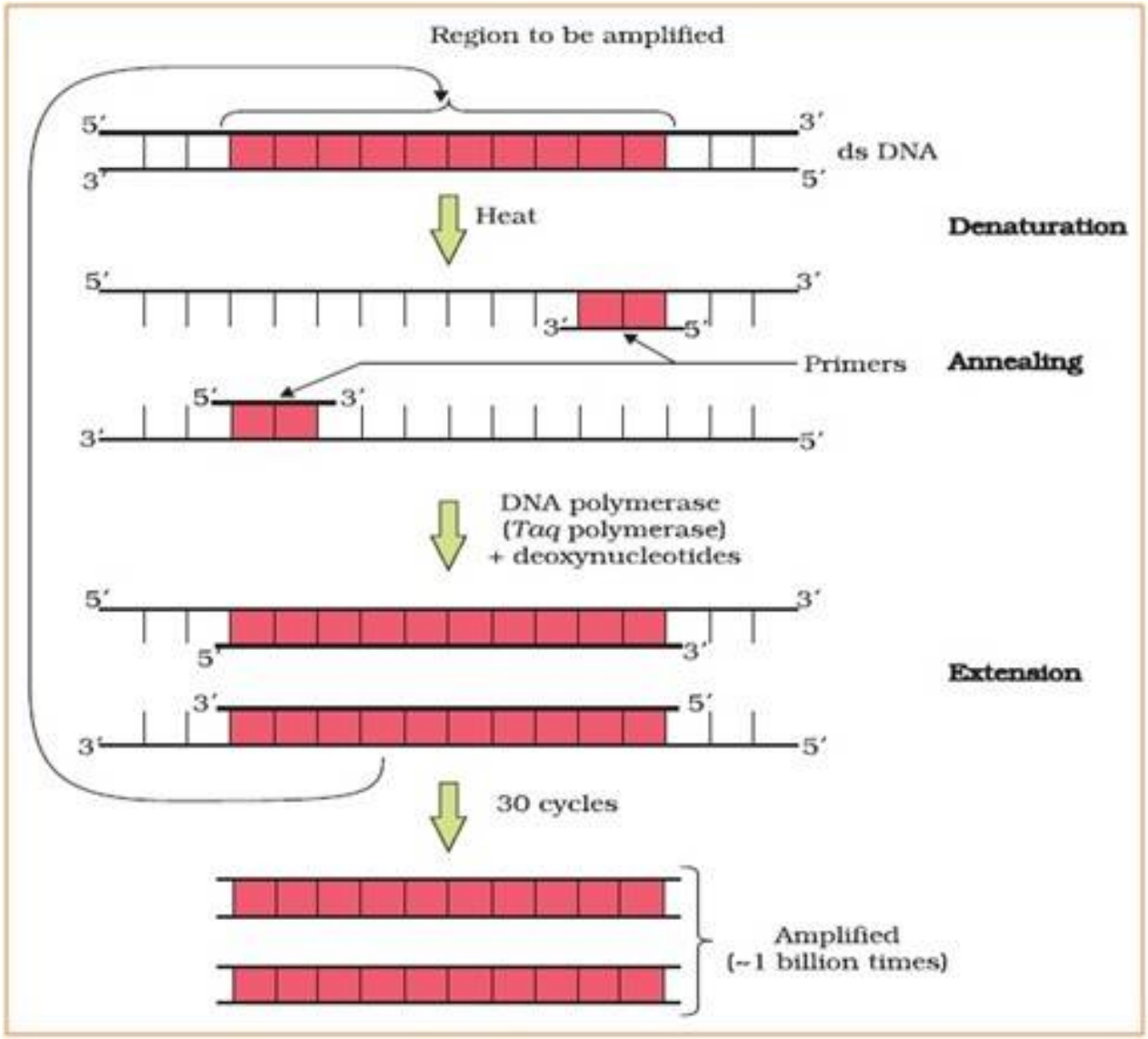
A typical agarose gel electrophoresis showing migration of undigested (lane 1) and digested set of DNA fragments lange 2 to 4)

- ✓ **DNA fragments separate according to their size through sieving effect ,provided by gel**
- ✓ **Smaller the fragment size farther it moves**
- ✓ **Separated DNA fragments visualized – after staining with “ethidium bromide”, followed by exposure to UV radiation (bright orange coloured bands of DNA)**
- ✓ **Elution – separated bands of DNA cut out from gel and extracted from gel piece**
- ✓ **Processes is repeated with vector DNA also**
- ✓ **Source DNA and vector DNA, cut with same RE are joined with ligase (rDNA formed)**



DNA that separates out can be removed by spooling

4. **Amplification of gene of interest using PCR**
   - ✓ **Polymerase chain reaction**
   - ✓ **Developed by Kary Mullis**
   - ✓ **Multiple copies of gene (or DNA) of interest synthesized in vitro using two sets of primers and enzyme DNA polymerase**
   - ✓ **Primers – small chemically synthesized oligonucleotides, complimentary to regions of DNA**
   - ✓ **Thermostable DNA plolymerase/Taq plymerase (isolated thermus aquaticus bacterium) remains active during high temperature induced de naturation of ds DNA**

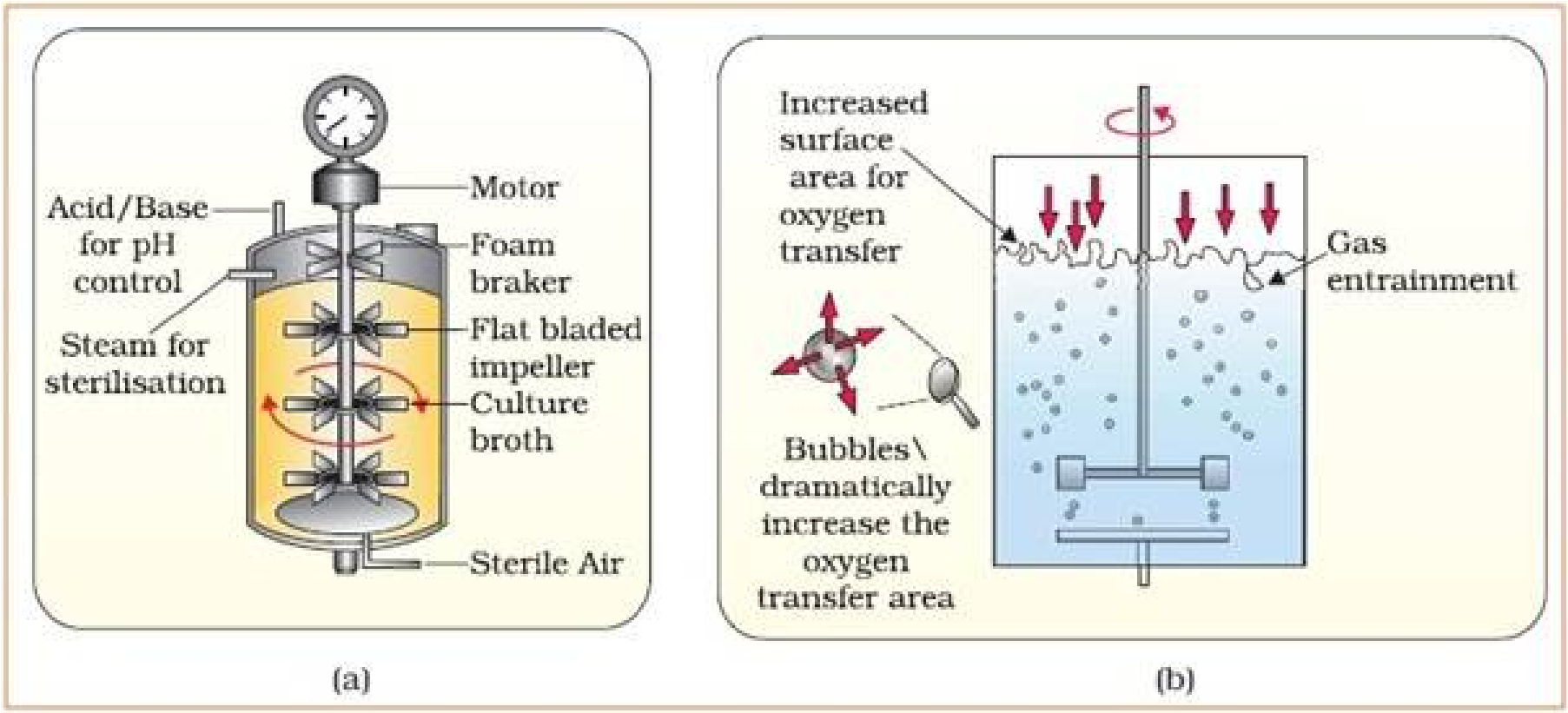


Polymerase chain reaction (PCR) : Each cycle has three steps: (i) Denaturation; (ii) Primer annealing; and (iii) Extension of primers

- ✓ **DNA polymerase can only add new nucleotides to 3'-OH end of growing strands (cannot begin synthesis de novo)**
- ✓ **Steps – denaturation, annealing, primer extention-polymerisation**
- ✓ **One billion copies made at end of 30 PCR cycles**
- ✓ **After "n"number of cycle – "2^n"molecules generated**

5. **Application of PCR – D_x of pathogens/ specific mutation/ prenatal diagnosis/ specific microorganisms/ DNA finger printing/ palaeontology**

6. **Insertion of rDNA into host cell**
7. **Culturing host cell in a nutrient medium at large scale for obtaining the foreign gene product**
   - ✓ **Ultimate aim – to produce desired protein (need for rDNA to be expressed)**
   - ✓ **Foreign gene gets expressed under appropriate condition**
   - ✓ **If any protein encoding gene is expressed in heterologous host – recombinant protein**
   - ✓ **Cells harbouring cloned genes of interest may be grown on small scale in lab, can not yield appreciable quantities of products**
   - ✓ **Bioreactors - large volumes (100-1000 litres) of culture can be processed**



(a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

- ✓ **Provides optimal growth conditions (temp., pH, substrate, salts, vitamins, oxygen)**
- ✓ **Most commonly used bioreactors – stirring type**
- ✓ **Cylindrical /curved base – fascilitated mixing of reactor contents**
- ✓ **Stirrir – fascilitates even mixing and oxygen availability throughout bioreactor. Alternately air can be bubbled .**
- ✓ **Has agitator system ,oxygen delivery system, foam controlled system, temperature control system, pH control system and sampling ports (small volumes of culture periodically withdrawn)**

- ✓ **Cells are maintained in their physiologically active log/ exponential phase**

8. **Downstream processes**
   - ✓ **After biosynthetic stage, separationand purification of product before it is ready marketing.**
   - ✓ **Suitable preservatives/ clinical trials (drugs)/ strict quality control testing**
   - ✓ **Vary from product to product**